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| 14. ABSTRACT<br><br>SRC-3Δ4, an N-terminus deletion isoform of steroid receptor coactivator (SRC-3), was shown to act as a signaling adaptor of EGF signaling in activating FAK. Its role in prostate cancer (PCa) progression is unclear. Interestingly, we found that SRC-3Δ4 is upregulated in castration resistant PCa cells as compared to androgen-dependent PCa cells. As such, we determined whether SRC-3Δ4 coactivates AR in an androgen-independent manner in response to EGF signaling. We have found that EGF stimulated the interaction of SRC-3Δ4 with AR and SRC-3Δ4 nuclear localization in androgen-depleted culture conditions. In response to EGF stimulation, SRC-3Δ4 was recruited to AR target genes' promoters and regulated AR target genes' transcription in an AR-dependent manner. Taken together, these results demonstrate that SRC-3Δ4 acts as a coactivator of AR in the nucleus and regulates the transcription of AR target genes in response to EGF signaling. |                  |                          |                            |                                                           |                                           |
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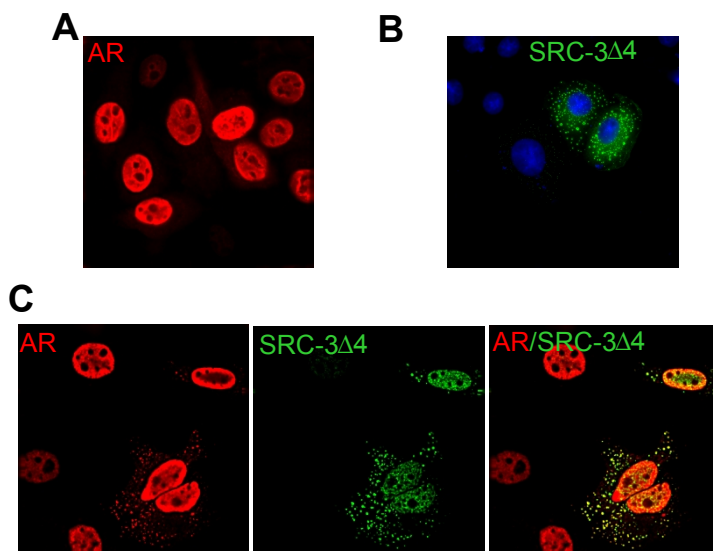
## INTRODUCTION

AR and its target genes are commonly expressed in and are believed to contribute to castration resistant prostate cancers (CRPC) (1, 2). Upregulation of growth factor signaling and alterations in AR coactivators are two major molecular mechanisms for castration-resistant tumor progression (3). Steroid receptor coactivator 3 (SRC-3) promotes prostate cancer cell growth and invasion and is important for tumor metastasis in animal models of prostate cancer (4-6). The importance of SRC-3 in castration-resistant prostate tumor progression, however, remains largely unknown. SRC-3 $\Delta$ 4, an N-terminus deletion isoform of SRC-3, was shown to act as a signaling adaptor of EGF signaling in activating FAK. Interestingly, our preliminary data (in the original application) showed that SRC-3 $\Delta$ 4 is upregulated in castration resistant PCa cells as compared to androgen-dependent PCa cells, and SRC-3 $\Delta$ 4 coactivates AR-mediated PSA-luciferase reporter activity in the absence of androgen. As such, we hypothesized that SRC-3 $\Delta$ 4 acts as a coactivator of AR in the nucleus and regulates the transcription of AR target genes in response to EGF signaling. The goal of **Aim1** in our Statement of Work is to determine whether SRC-3 $\Delta$ 4 coactivates AR in an androgen-independent manner in response to EGF signaling, which is the main task for the first-year period.

## BODY

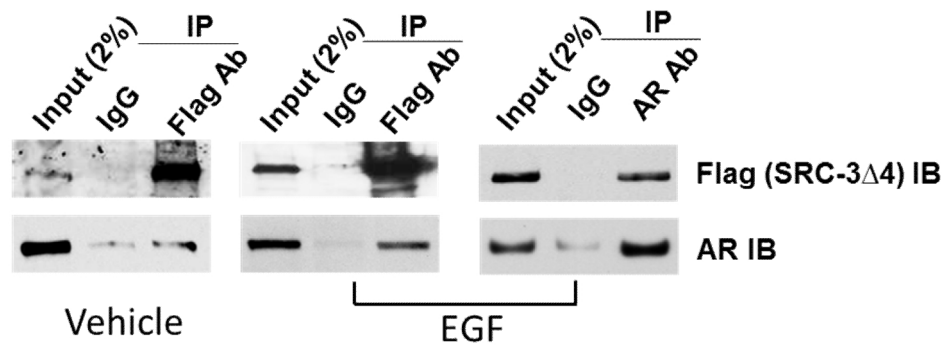
**Task 1(Aim 1)** is to test whether SRC-3 $\Delta$ 4 coactivates AR in an androgen-independent manner in response to EGF signaling. We decided to address this Aim by performing the following sub-tasks.

**Task 1a** To determine whether SRC-3 $\Delta$ 4 interacts with AR and translocates to the nucleus upon EGF stimulation. In comparison with full-length SRC-3 protein, SRC-3 $\Delta$ 4 lacks the N-terminal bHLH-PAS region that contains the nuclear localization signal (NLS) (7). There is an intriguing possibility, however, that SRC-3 $\Delta$ 4 interacts with AR via its steroid receptor interacting domain and translocates to the nucleus together with AR. We analyzed the subcellular localization and the interaction of SRC-3 $\Delta$ 4 and AR by immunofluorescence and co-immunoprecipitation (co-IP) in LNCaP-C4-2, a CRPC cell line. AR primarily localizes in the nucleus and a small fraction of AR is shown in the cytoplasm (Fig. 1A). As there is not a specific antibody available for differentiating endogenous SRC-3 $\Delta$ 4 from the full-length SRC-3, we transiently transfected SRC-3 $\Delta$ 4 with a Flag tag into LNCaP-C4-2 and detected SRC-3 $\Delta$ 4 using a Flag antibody. As shown in Fig. 1B, SRC-3 $\Delta$ 4 primarily localizes in the cytoplasm in the absence of EGF stimulation. In contrast, SRC-3 $\Delta$ 4 is shown to localize in the nucleus and co-localizes with AR (Fig. 1C) in response to EGF stimulation. Interestingly, SRC-3 $\Delta$ 4 and AR are also shown to co-localize in the cytoplasm (Fig. 1C), implying that EGF stimulates the interaction of SRC-3 $\Delta$ 4 with AR in the cytoplasm and its translocation with AR into the nucleus.



**Figure 1 EGF stimulates SRC-3 $\Delta$ 4's interaction with AR and its nuclear localization.** (A). Endogenous expression and cellular localization of AR in LNCaP-C4-2 cells growing in phenol-red free RPMI medium containing 5% charcoal-stripped fetal bovine serum (cs-FBS). (B). Cellular localization of SRC-3 $\Delta$ 4 in LNCaP-C4-2 cells transfected with a construct expressing SRC-3 $\Delta$ 4 bearing a Flag-tag at the C-terminus (SRC-3 $\Delta$ 4Flag). (C). LNCaP-C4-2 cells were cultured in phenol-red free RPMI medium containing 5% cs-FBS. Cells were transiently transfected with a construct expressing SRC-3 $\Delta$ 4Flag. 24 hours post-transfection, cells were serum-starved for 24 hours, and then were stimulated with 50 ng/ml EGF for 30 minutes. SRC-3 $\Delta$ 4Flag and endogenous AR proteins were detected by immunofluorescence using a Flag antibody (Sigma) and an AR antibody (Santa Cruz), respectively. Protein subcellular localization and co-localization were analyzed by deconvolution microscopy. The nucleus was stained with DAPI in blue color.

We confirmed the interaction between SRC-3Δ4 and AR by reciprocal co-IP using either a Flag antibody (against SRC-3Δ4) or an AR antibody. As shown in Fig. 2, EGF greatly stimulates the interaction of SRC-3Δ4 with AR under androgen-depleted culture conditions in LNCaP-C4-2 cells.



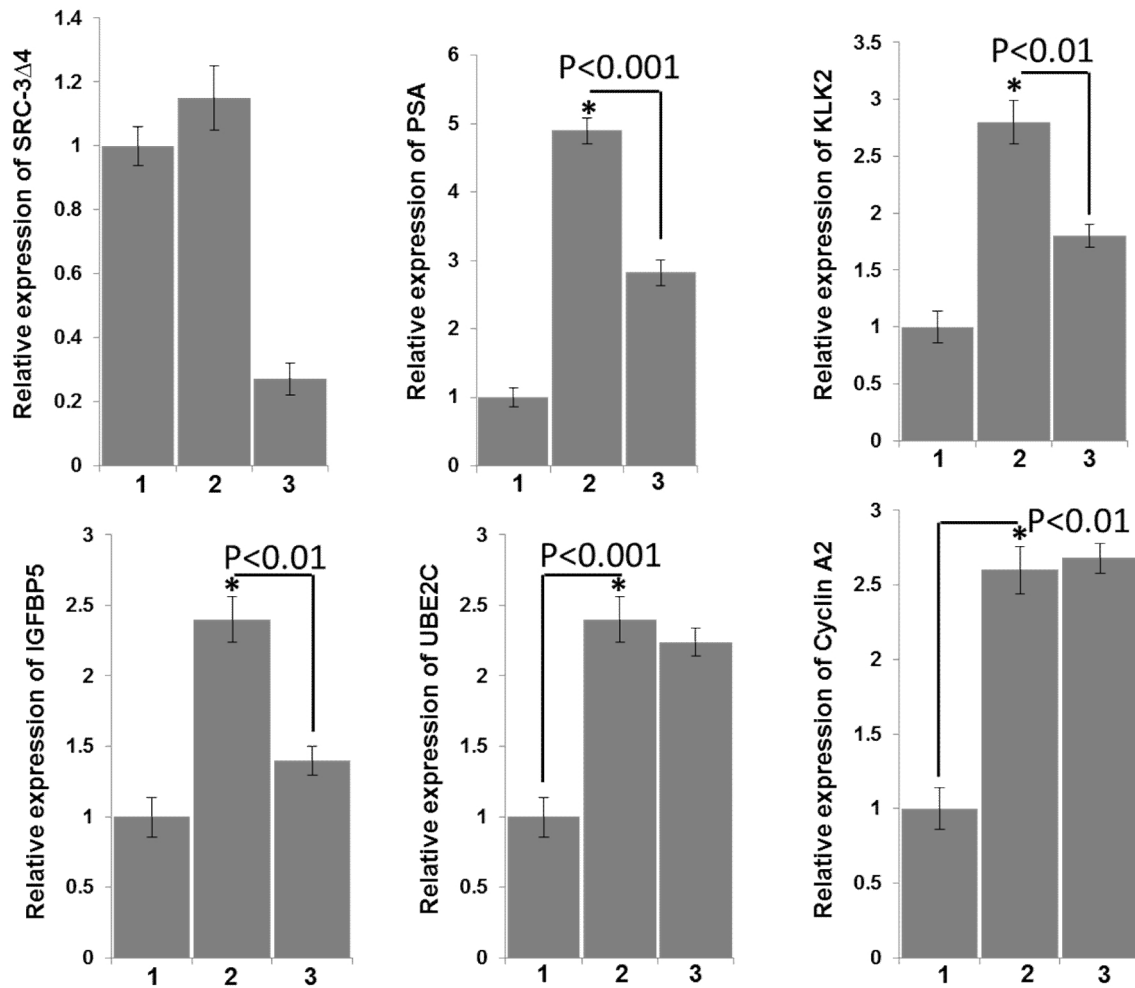
**Figure 2 EGF stimulates the interaction of SRC-3Δ4 with AR.** LNCaP-C4-2 cells were cultured in phenol-red free RPMI medium containing 5% cs-FBS. Cells were transiently transfected with a construct expressing SRC-3Δ4Flag. 24 hours post-transfection, cells were serum-starved for 24 hours, and then were stimulated with 50 ng/ml EGF or vehicle for 30 minutes, then lysed. The interaction between SRC-3Δ4 and AR was analyzed by immunoprecipitation (IP) using a Flag antibody, an AR antibody, or a control IgG from total cell lysates, followed by Western blotting. Ab: antibody. IB: immunoblots.

**Task 1b** To determine whether SRC-3Δ4 regulates AR target gene expression upon EGF stimulation. After revealing an interaction and co-localization between SRC-3Δ4 and AR in the nucleus, we then tested whether SRC-3Δ4 regulates AR target gene expression upon EGF stimulation. The following genes have been shown to be regulated by AR and are important for androgen-independent or castration resistant prostate cancer growth and invasion and were analyzed in our experiment: PSA (8), kallikrein-related peptidase 2 (KLK2) (9), cyclin A2 (4), ubiquitin-conjugating enzyme E2C (UBE2C) (10), insulin-like growth factor binding protein 5 (IGFBP5) (11), and TMPRSS2-ERG fusion gene (12). As shown in Fig. 3, knockdown of SRC-3Δ4 by siRNA significantly decreased EGF-stimulated expression of PSA, KLK2, and IGFBP5, but had no significant effect on UBE2C and cyclin A2, suggesting SRC-3Δ4 regulates a subset of AR target genes. To determine the role of SRC-3Δ4 on TMPRSS2-ERG fusion gene expression, SRC-3Δ4Flag was stably transduced into VCap cells (Fig. 4A). While knockdown of AR (Fig. 4B) greatly reduced the expression of TMPRSS2-ERG fusion gene, neither EGF stimulation nor SRC-3Δ4 overexpression significantly altered TMPRSS2-ERG fusion gene expression (Fig. 4C).

**Task 1c** To determine by chromatin immunoprecipitation (ChIP) assay whether EGF stimulates the recruitment of SRC-3Δ4 and AR to AR target genes. Steroid receptor coactivators regulate gene expression by binding to steroid receptors bound on the target gene promoter. To determine whether EGF stimulates the recruitment of SRC-3Δ4 to AR target genes, we generated an LNCaP-C4-2 cell pool stably expressing SRC-3Δ4Flag by lentiviral transduction (Fig. 5A). Occupancy of SRC-3Δ4 on the gene promoters were determined by ChIP assay using a Flag antibody. Interestingly, even in the absence of EGF stimulation, there is detectable occupancy of SRC-3Δ4 on PSA gene promoter (Fig. 5C). Importantly, in comparison with the vehicle treatment (Fig. 5C), EGF stimulation greatly enhanced the occupancy of SRC-3Δ4 on PSA promoter (Fig. 5D). To determine whether the recruitment of SRC-3Δ4 onto the PSA promoter is dependent on AR, we depleted AR by RNA interference (Fig. 5B). Depletion of AR virtually abolished the occupancy of SRC-3Δ4 on PSA gene promoter (Fig. 5C and 5D). Similar results were observed on the occupancy of SRC-3Δ4 on IGFBP5 gene promoter (Fig. 5F) except that there was no detectable recruitment of SRC-3Δ4 in the absence of EGF stimulation (Fig. 5E).

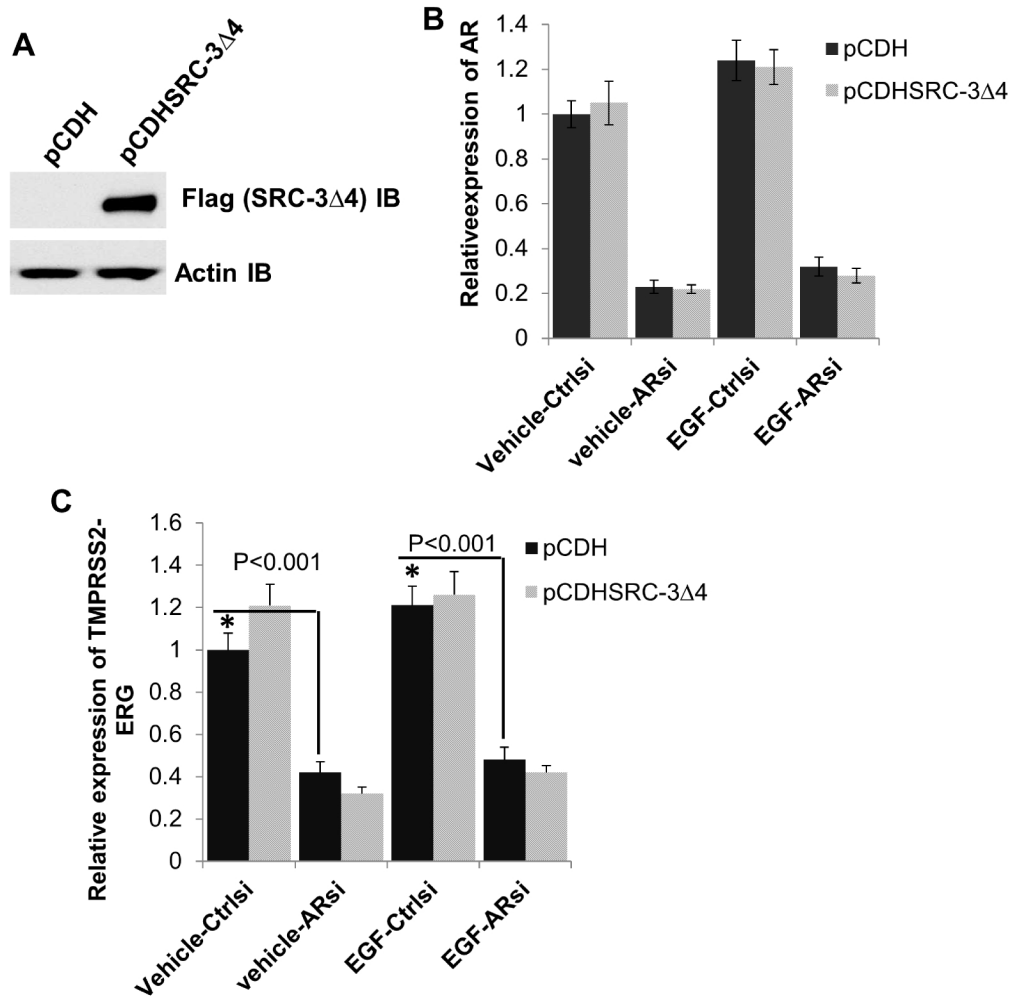
A CHIP-reCHIP sequencing was proposed to identify novel genes that may be transactivated by AR/SRC-3Δ4 complex upon EGF stimulation. Due to technical difficulty with CHIP using an AR antibody, we have not

yet successfully fulfilled this assay. We are trying different AR antibodies and are optimizing experimental conditions.

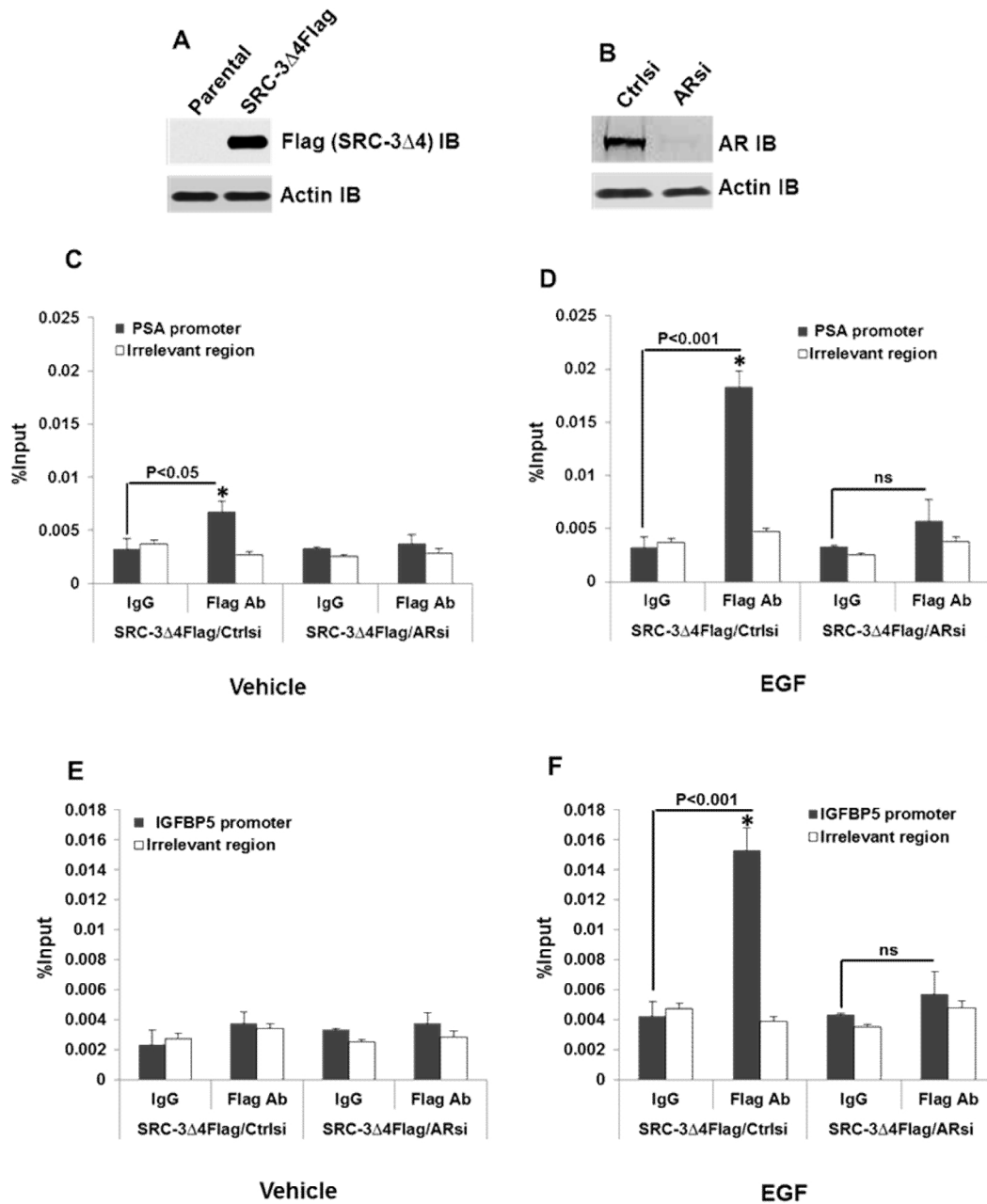


Labels on the X-axis: **1.** Ctrlsi-Vehicle, **2.** Ctrlsi-EGF, and **3.** SRC-3Δ4si-EGF

**Figure 3 SRC-3Δ4 regulates the expression of a subset of AR target genes that are stimulated by EGF.** LNCaP-C4-2 cells were cultured in phenol-red free RPMI medium containing 5% cs-FBS. Cells were transiently transfected with either a siRNA specifically targeting only SRC-3Δ4 (SRC-3Δ4si) or a non-targeting control siRNA (Ctrlsi). 24 hours post-transfection, cells were serum-starved for 24 hours, followed by the stimulation with 50 ng/ml EGF or vehicle for 24 hours. Expression of SRC-3Δ4, GAPDH, and AR target genes including PSA, KLK2, IGFBP5, Cyclin A2, and UBE2C was determined by RT-qPCR analysis. Data are presented using the comparative Ct method, in which GAPDH mRNA was used as the normalizer, and the normalized Ctrlsi-Vehicle treated samples were set to unity. Values represent the means  $\pm$  SE of three separate experiments. “\*” indicates a significant difference (Student’s *t* test).



**Figure 4 SRC-3Δ4 is not involved in the regulation of TMPRSS2-ERG by AR. (A).** Generation of VCaP cell pool stably expressing SRC-3Δ4Flag. VCaP cells were transduced with lentiviruses expressing either SRC-3Δ4Flag or the empty vector pCDH. Two days post-transduction, cells were split and selected by puromycin (1 μg/ml). Expression of SRC-3Δ4Flag in the stable cell pools was analyzed by Western blotting using a Flag antibody. β-actin was probed as a loading control. **(B) and (C).** VCaP stable cell pools were cultured in phenol-red free RPMI medium containing 5% cs-FBS. Cells were transiently transfected with either a siRNA specifically targeting AR (ARsi) or a non-targeting control siRNA (Ctrl si). 24 hours post-transfection, cells were serum-starved for 24 hours, and then were stimulated with 50 ng/ml EGF or vehicle for 24 hours. Expression of AR **(B)**, GAPDH, and TMPRSS2-ERG **(C)** was determined by RT-qPCR analysis. Data are presented using the comparative Ct method, in which GAPDH mRNA was used as the normalizer, and the normalized Vehicle-Ctrl si sample of pCDH cell pool was set to unity. Values represent the means ± SE of three separate experiments. “\*” indicates a significant difference (Student’s *t* test).



**Figure 5** EGF stimulates SRC-3Δ4's occupancy on the AR target genes' promoters in an AR-dependent manner. **(A)**. Generation of LNCaP-C4-2 cell pool stably expressing SRC-3Δ4Flag. LNCaP-C4-2 cells were transduced with lentiviruses expressing SRC-3Δ4Flag. Two days post-transduction, cells were split and selected by puromycin (1 μg/ml). Expression of SRC-3Δ4Flag in the stable cell pools was analyzed by Western blotting using a Flag antibody. β-actin was probed as a loading control. **(B)**. Transient knockdown of AR in LNCaP-C4-2 cell pool stably expressing SRC-3Δ4Flag. Cells were cultured in phenol-red free RPMI medium containing 5% cs-FBS. Cells were transiently transfected with either a siRNA specifically targeting AR (ARsi) or a non-targeting control siRNA (Ctrl si). 24 hours post-transfection, cells were serum-starved for 24 hours, and then were stimulated with 50 ng/ml EGF or vehicle for 24 hours. Knockdown of AR was confirmed by Western blotting using an AR antibody. **(C) and (D)**. SRC-3Δ4's occupancy on PSA gene promoter. LNCaP-C4-2 cells stably expressing SRC-3Δ4Flag were transiently transfected with either a siRNA specifically targeting AR (ARsi) or a non-targeting control siRNA (Ctrl si) as shown in **(B)**. ChIP assays then were performed using either a Flag Ab or mouse IgG. SRC-3Δ4Flag protein occupancy on PSA promoter region in response to vehicle **(C)** or EGF **(D)** stimulation was analyzed by quantitative real-time PCR and presented as the percentage of sheared chromatin input. An irrelevant region (1800 bp downstream of transcription start site) was served as a negative control. **(E) and (F)**. ChIP analysis of SRC-3Δ4's occupancy on IGFBP5 gene promoter in response to vehicle **(E)** or EGF **(F)**. Values represent the means ± SE of three separate experiments. "\*" indicates a significant difference (Student's *t* test), and "ns" means no significant difference.



**Task 2 (Aim 2)** is to determine the role of SRC-3Δ4 in androgen-independent prostate cancer cell growth and invasion.

EGF signaling activates AR and stimulates prostate cancer cell growth and invasion. Our findings in Aim 1 suggest that in response to EGF stimulation, SRC-3Δ4 regulates the expression of AR target genes including PSA, KLK2, and IGFBP5. These genes are important for castration-resistant prostate cancer cell growth and/or invasion. To determine the role of SRC-3Δ4 in castration-resistant prostate cancer cell growth and invasion, we depleted endogenous SRC-3Δ4 in two androgen-independent prostate cancer cell lines CWR22Rv1 and LNCaP-C4-2 by stably expressing a shRNA specifically targeting SRC-3Δ4 (Fig. 6). The effect of SRC-3Δ4 depletion on the growth and invasion of CWR22Rv1 and LNCaP-C4-2 cells is being tested by MTS cell growth assay and matrigel-transwell cell invasion assay, respectively.



**Figure 6 Generation of LNCaP-C4-2 and CWR22Rv1 cell pools with stable knockdown of SRC-3Δ4.** LNCaP-C4-2 and CWR22Rv1 were transduced with lentiviruses expressing either a shRNA specifically targeting SRC-3Δ4 (shSRC-3Δ4) or a scrambled shRNA sequence (shCtrl). Two days post-transduction, cells were split and selected by puromycin (1 μg/ml). Expression of SRC-3Δ4 in the stable cell pools was analyzed by Western blotting using a SRC-3 antibody that recognizes both SRC-3 and SRC-3Δ4. β-actin was probed as a loading control.

## KEY RESEARCH ACCOMPLISHMENTS

1. Demonstrated that SRC-3Δ4 interacts with AR and translocates to the nucleus upon EGF stimulation.
2. Demonstrated that SRC-3Δ4 regulates AR target gene expression upon EGF stimulation.
3. Demonstrated that EGF stimulates the recruitment of SRC-3Δ4 to AR target genes in an AR-dependent manner.

## REPORTABLE OUTCOMES

1. Generated letiviruses expressing SRC-3Δ4Flag or the pCDH empty vector following the manufacturer's instructions (System Biosciences).
2. Generated VCaP cell pools stably expressing SRC-3Δ4Flag or the pCDH empty vector by lentiviral transduction with lentiviruses expressing either SRC-3Δ4Flag or the empty vector pCDH, respectively.
3. Generated lentiviruses expressing a shRNA specifically targeting SRC-3Δ4 (shSRC-3Δ4, 7) or a scrambled shRNA sequence (shCtrl) using the pSIH-H1 shRNA-expressing system (System Biosciences).
4. Generated LNCaP-C4-2 and CWR22Rv1 cell pools stably expressing either shSRC-3Δ4 or shCtrl.

- Submitted an abstract entitled “SRC-3Δ4 coactivates AR target gene expression in an androgen-independent manner upon EGF stimulation in prostate cancer cells.” to an AACR special conference “Tumor Invasion and Metastasis” that will be held in January, 2001 in San Diego.

## CONCLUSION

Our results suggest that SRC-3Δ4 coactivates AR target gene expression in an androgen-independent manner upon EGF stimulation.

**So What:** The findings obtained during the first year corroborate our hypothesis that SRC-3Δ4 regulates AR target gene expression in an androgen-independent manner upon EGF stimulation, which lays an important base for us to further elucidate the underlying molecular mechanisms and the role of SRC-3Δ4 in castration resistant tumor growth and progression in the next two years.

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